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DNA sequence analysis of conserved genes reveals hybridization events that increase genetic diversity in *Verticillium dahliae*

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ABSTRACT

The hybrid origin of a *Verticillium dahliae* isolate belonging to the vegetative compatibility group (VCG) 3 is reported in this work. Moreover, new data supporting the hybrid origin of two *V. dahliae* var. *longisporum* (VDLSP) isolates are provided as well as information about putative parentals. Thus, isolates of VDLSP and *V. dahliae* VCG3 were found harboring multiple sequences of actin (*Act*), β-tubulin (*β-tub*), calmodulin (*Cal*) and histone 3 (*H3*) genes. Phylogenetic analysis of these sequences, the internal transcribed sequences (ITS-1 and ITS-2) of the rRNA genes and of a *V. dahliae*-specific sequence provided molecular evidences for the interspecific hybrid origin of those isolates. Sequence analysis suggests that some of VDLSP isolates may have resulted from hybridization events between a *V. dahliae* isolate of VCG1 and/or VCG4A and, probably, a closely related taxon to *Verticillium albo-atrum* but not this one. Similarly, phylogenetic analysis and PCR markers indicated that a *V. dahliae* VCG3 isolate might have arisen from a hybridization event between a *V. dahliae* VCG1B isolate and as yet unidentified parent. This second parental probably does not belong to the *Verticillium* genus according to the gene sequences dissimilarities found between the VCG3 isolate and *Verticillium* spp. These results suggest an important role of parasexuality in diversity and evolution in the genus *Verticillium* and show that interspecific hybrids within this genus may not be rare in nature.

Introduction

The genus *Verticillium* has traditionally comprised a group of soilborne fungal species (i.e. *V. albo-atrum*, *V. dahliae*, *V. tricorpus*, *V. nigrescens*, *V. nubilum*, and *V. theobromae*) of which *V. albo-atrum* and *V. dahliae* are the most important because of their wide distribution, broad host range and the severe wilt disease they cause in many economically important crop plants (Pegg & Brady 2002). It should be noted that *V. nigrescens* and *V. theobromae* have recently been assigned to different genera by Zare *et al.* (2007). All these species are haploid, strict asexually-reproducing for which no sexual state has been identified to date (Pegg & Brady 2002). Nevertheless, a parasexual cycle may be operating in nature between species within this genus, as indicated by results from *in vitro* assays, thus providing the benefits of genetic

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recombination to *Verticillium* (Hastie 1964; Heale 1988; Karapapa *et al.* 1997). However, the extent to which the parasexual cycle occurs in nature is not known. Because of the strict asexual reproduction in *Verticillium* spp. hyphal anastomosis is a prerequisite to somatic hybridization and eventual parasexual genetic recombination among different isolates of the pathogen (Hastie 1981; Pegg & Brady 2002). Somatic hybridization exists among different *V. dahliae* isolates, which are then grouped in different vegetative compatibility groups (VCGs) according to the ability of isolates within a VCG to undergo hyphal anastomosis and form stable heterokaryons (Leslie 1993; Katan 2000). Currently, eight VCGs (VCG1A, VCG1B, VCG2A, VCG2B, VCG2Ba, VCG3, VCG4A, VCG4B, and VCG6) have been identified in *V. dahliae* by complementation assays with nitrate-nonutilizing (*nit*) mutants of isolates from diverse hosts and geographic origin worldwide (Joaquim & Rowe 1990; Strausbaugh *et al.* 1992; Bell 1994; Chen 1994; Katan 2000; Bhat *et al.* 2003; Jiménez-Díaz *et al.* 2006).

In 1961, a *Verticillium* strain producing conidia approximately twice as long as those of *V. dahliae* was isolated from wilted horseradish (Stark 1961), and named *V. dahliae* var. *longisporum* (VDLSP). Later, other isolates with similar characteristics were isolated (mainly from crucifers) and included in this group (Puhalla & Hummel 1983; Jackson & Heale 1985; Subbarao *et al.* 1995; Karapapa *et al.* 1997; Fahleson *et al.* 2003). In 1997, Karapapa *et al.* proposed to erect VDLSP as a new species, namely *V. longisporum*. This was based on distinctive morphological, molecular and virulence characteristics (e.g. longer conidia, elongate microsclerotia, near double DNA quantity, infecting crucifers mainly, etc.) compared to *V. dahliae*. In addition, several studies have demonstrated molecular differences between VDLSP and *V. dahliae* isolates, either using RAPD (Zeise & von Tiedemann 2002b) or AFLP fingerprinting (Collins *et al.* 2003; Fahleson *et al.* 2004; Collado-Romero *et al.* 2006). Nevertheless, during the last 10 y, a high level of intraspecific diversity has been identified within isolates of VDLSP, giving rise to the controversy on the proper taxonomic status of *V. longisporum vs V. dahliae* var. *longisporum* (Karapapa *et al.* 1997; Zeise & von Tiedemann 2001; Barbara & Clewes 2003; Collins *et al.* 2003; 2005; Pantou *et al.* 2005). Thus, some authors do not consider appropriate the newly erected species status for VDLSP isolates, mainly due to the lack of homogeneity within the group. Therefore, because of their higher nuclear DNA content and conidial length *Verticillium* isolates in this group are often referred as ''long-spored'', ''near diploid'' or ''amphihaploid'' (Barbara & Clewes 2003; Collins *et al.* 2003; Qin *et al.* 2006). Moreover, according to the molecular diversity (AFLP fingerprinting) found within some VDLSP isolates, Collins *et al.* (2003) divided them into VDLSP α and β groups. It has been suggested that long-spored isolates are the result of a hybridization event between *V. dahliae* and *V. albo-atrum* (Karapapa *et al.* 1997; Collins *et al.* 2003; Qin *et al.* 2006) or between *V. dahliae* and another as yet unidentified species (Clewes *et al.* 2008). Although for the last 15 y *V. dahliae* and *V. albo-atrum* have been pointed out as the parental species of VDLSP isolates, molecular data have not yet established this fact unequivocally. Interestingly, VDLSP isolates share similarities with *V. dahliae* and *V. albo-atrum* but also bear species-specific characteristics such as host preference (Messner *et al.* 1996; Karapapa *et al.* 1997; Karapapa & Typas 2001; Zeise & von Tiedemann 2001, 2002a; Stevenson *et al.* 2002; Fahleson *et al.* 2003), which suggest a possible role for polyploidy within the genus *Verticillium*. This is particularly interesting since fungal interspecific hybrids seems to be more frequent and play more important roles in nature than previously thought (Schardl & Craven 2003; Clewes & Barbara 2007; Selosse & Schardl 2007).

Recent studies have tried to enlighten the origin of VDLSP hybrid isolates by comparative analysis of DNA sequences such as the β-*tubulin* gene, the 5S rRNA-associated sequence, or the complete intergenic spacer region of the nuclear ribosomal RNA gene (Qin *et al.* 2006; Clewes *et al.* 2008). Moreover, Clewes *et al.* (2008) reported the presence of two different alleles for the β-*tubulin* gene in long-spored isolates, supporting their hybrid origin, but not in *V. dahliae* haploid isolates. In a recent review, Klosterman *et al.* (2009) highlighted the controversy on the appropriate classification of VDLSP isolates, and pointed out that unavailability of sequences from these isolates makes it difficult to carry out comparative genetic analyses.

Recently, we have demonstrated that analyses of AFLP fingerprinting and specific DNA sequences of the *V. dahliae* genome is an excellent approach to unravel phylogenetic relationships at the intraspecific level in *V. dahliae*, i.e. among VCGs (Collado-Romero *et al.* 2008). In the present study we aimed to: *i*) determine the genetic relationship that might exist among VDLSP isolates, all *V. dahliae* VCGs (including VCG3 that was not included in previous studies), *V. albo-atrum* and *V. nigrescens*; and *ii*) provide molecular evidences that support the interspecific hybrid nature of VDLSP isolates and uncover the identity of their putative parentals. For that purpose, we used a set of isolates representative of diverse hosts and geographic origins from which sequences of five conserved DNA regions: actin (*Act*), calmodulin (*Cal*), β-tubulin (β-*tub*), histone 3 (*H3*), and ITS 1 and 2 of the rDNA were analyzed. We provide evidences that occurrence of interspecific hybrids within the *Verticillium* genus may not be a rare phenomenon in nature.

Materials and methods

Verticillium *spp. isolates and fungal DNA purification*

A set of *Verticillium* spp. isolates from diverse host source and geographic origin was use for this study (Table 1). This includes a *Verticillium nigrescens* isolate, a *Verticillium albo-atrum* isolate, two *Verticillium dahliae* var. *longisporum* (VDLSP) isolates, and 15 *V. dahliae* isolates representative of all VCGs currently identified. Single-spore cultures of all isolates are deposited in the culture collection of the Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, CSIC, Córdoba, Spain. Active cultures of isolates were obtained on water agar amended with chlorotetracycline (0.3 g l^{-1}) (Sigma–Aldrich, St. Louis, MO) and subsequent subculturing on plum-lactose-yeast agar (PLYA). DNA was extracted from mycelia obtained from 6- to 7-day-old cultures of isolates in Czapeck-Dox broth (Difco Laboratories, Detroit, MI, USA), according to Collado-Romero *et al.* (2006). DNA purity

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Table 1 – Isolate code, geographic origin, host plant, VCG, pattern of PCR markers and *Verticillium dahliae*-species-specific sequence type

Isolates[a]	Geographic origin	Host	VCG[b]	PCR pattern[c]	*Verticillium dahliae* sequence type[d]
V. dahliae					
V138I	Spain	*Gossypium hirsutum*	1A	A	4
V607I (R04)	USA	*Fraxinus pennsylvanica*	1B	B	4
V320I	California	*G. hirsutum*	2A	C	2
V800I	Spain	*Olea europaea*	2A	C	2
V720I (V39)	Italy	*O. europaea*	2A	C	2
V702I	Spain	*Cynara cardunculus*	2Ba	B	4
V613I	Spain	*C. cardunculus* var. *scolymus*	2B	B	4
V357I (JY)	China	*G. hirsutum*	2B	D	1
V396I	Spain	*C. cardunculus*	2B	D	1
V510I (tom20)	Israel	*Lycopersicon esculentum*	2B	D	1
70–21	California	*Capsicum annuum*	3	B	6
131-M	USA	*Solanum tuberosum*	4A	E	7
V304I (cot120)	Israel	*G. hirsutum*	4B	C	2
V684I	Spain	*C. cardunculus*	4B	C	2
V560I (VdCa.83a)	California	*C. annuum*	6	C	1
V. dahliae var. *longisporum*					
Vd-1 (V558I)	Sweden	*Brassica napus* ssp. *oleifera*	–	NA	3
90-10 (V559I)	California	*B. oleracea* var. *botrytis*	–	NA	3
Verticillium albo-atrum					
V48	UK	*Humulus lupulus* L.	–	NA	–
Verticillium nigrescens					
V51	UK	*S. tuberosum* L.	–	NA	–

a Isolate code. In brackets, isolate code used by the provider or in previous studies.
b VCG assessment of the isolates was reported earlier (Collado-Romero *et al.* 2006, 2008; Korolev *et al.* 2000; Korolev *et al.* 2001; R. Rowe, personal communication; Bhat *et al.* 2003; and N. Korolev, J. Katan, R.M. Jiménez-Díaz y T. Katan, unpublished data).
c Differential PCR pattern amplification according to Collado-Romero *et al.* 2009. A = 462 bp (+), 334 (+), 688 (–), 964 (–); B = 462 bp (–), 334 (+), 688 (–), 964 (–); C = 462 bp (–), 334 (–), 688 (+), 964 (–); D = 462 bp (–), 334 (–), 688 (+), 964 (+); E = 462 bp (–), 334 (+), 688 (+), 964 (+); NA = none of these amplicons were amplified.
d Sequence (*seq*) type obtained after amplification with primer pair DB19/DB22. –, no amplification.

and concentration were determined spectrophotometrically using a Biophotometer (Eppendorff AG, Hamburg, Germany) and by agarose gel electrophoresis according to standard procedures (Sambrook *et al.* 1989). DNA solutions were stored at −20 °C until used.

Generation of AFLP profiles and binary matrixes

AFLP profiles of *Verticillium dahliae* VCG3 isolate 70-21 and VDLSP isolate Vd-1 were generated using the procedure described in Collado-Romero *et al.* (2006). AFLP profiles of the remaining isolates (Table 1) were available from two previous studies (Collado-Romero *et al.* 2006, 2008). The AFLP profile for VDLSP isolate 90-10 was produced again in this present work to assess the reproducibility and consistency of the AFLP procedure. AFLP profiles from all isolates were jointly analyzed using Genotyper software version 2.5 (Applied Biosystems). Only unambiguous peaks were scored for presence or absence (1 = presence or 0 = absence). All DNA fragments within the range of 50–490 bp were selected first using the 'unmark overlapping peaks' option and AFLP profiles were rescaled using the 'normalized scale option'. Unambiguous, consistent peaks, which scaled higher than 100 fluorescent units, were then selected and those that were inconsistent were deleted manually. A binary character matrix was developed by combining all data and used for subsequent phylogenetic analysis.

Phylogenetic analysis of AFLP patterns

The TREECON software (Van de Peer & de Wachter 1994) was used to develop a phylogenetic tree. Genetic distances were computed using the simple matching method (Sneath & Sokal 1973). The genetic distance matrixes were used to generate a phylogram using the neighbour-joining (NJ) method (Saitou & Nei 1987). A bootstrap analysis of 1000 permutations was used to test reliability of branches in the tree.

Amplification, cloning and sequencing of specific DNA sequences

Purified DNA from *Verticillium nigrescens*, *Verticillium dahliae* VCG3, and VDLSP isolates were used for the amplification and sequencing of *Act*, *Cal*, β-*tub*, and *H3* genes, as well as for the ITS-1 and ITS-2 regions of rDNA. For the other isolates used and described in Table 1 these sequences were available from a previous work (Collado-Romero *et al.* 2008). For the *V. dahliae* and VDLSP isolates, the *V. dahliae*-specific polymorphic DNA sequence (Mercado-Blanco *et al.* 2003; Collins *et al.*

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2005; Collado-Romero *et al.* 2008) was also amplified and sequenced using primer pair DB19/DB22 (Carder *et al.* 1994). Primers sequences, reaction mixtures and PCR cycling protocols used have been previously described (Collado-Romero *et al.* 2008). The amplification products (5 µl) were separated by electrophoresis on 1 % agarose gels using standard procedures (Sambrook *et al.* 1989). If only one band was identified in agarose gels, that was purified from the PCR mix using QIaquick PCR purification kit (Qiagen GmbH, Hilden, Germany). When two or more bands were identified in gels, they were purified independently using the QIAquick gel extraction kit (Qiagen). Direct sequencing of PCR products was performed at the Servicio de Secuenciación de ADN, Centro de Investigaciones Biológicas (CSIC, Madrid, Spain), using forward primers in all cases and forward and reverse primers occasionally. Sequences were edited with EditSeq (Lasergene, Madison, WI, USA). A search for sequence similarities was performed with the BLASTN v.2.2.10 program of the NCBI network service (Altschul *et al.* 1997). When sequencing chromatograms indicated mix of DNA sequences (revealed by unambiguous nucleotide positions in the chromatograms), PCR products were ligated to plasmid p-GEM®-T Easy using the p-GEM®-T Easy Vector System I (Promega Corporation, Madison, WI) according to the manufacturer's instructions. Electrocompetent *Escherichia coli* DH5α cells were transformed with the recombinant plasmids using a MicroPulser system (BioRad, Hercules, CA) according to the manufacturer's indications. Four white colonies per each transformation experiment were selected from LB plates amended with ampicillin (100 µg ml^{-1}), IPTG (0.5 mM) and X-GAL (80 µg ml^{-1}) (Sigma–Aldrich Co.). Plasmid DNA of each selected colony was extracted from 5 ml overnight cultures grown in LB broth at 37 °C using the QIAprep® Miniprep kit (Qiagen). Inserted sequences in plasmids were fully sequenced using the T7 promoter primer. All sequences have been deposited in the EMBL/GenBank/DDBJ nucleotide sequence databases.

Phylogenetic analysis of DNA sequences

The new sequences of the four genes (*Act*, *β-tub*, *Cal* and *H3*) and the ITS regions obtained in this work were used for phylogenetic analysis in combination with DNA sequences of *Verticillium dahliae* isolates representative of the different *V. dahliae* VCGs (e.g. VCGs 1A, 1B, 2A, 2B^{334}, 2B^{824}, 2Ba, 4A, 4B and 6) (Table 1), and *Verticillium albo-atrum* V-48 isolate sequenced in a previous work (Collado-Romero *et al.* 2008). The GenBank Accession Numbers are: *V. albo-atrum* (Act-V48I: DQ266104; Bt-V48I: DQ266147; V48I-cal: DQ266165; H3-V48I: DQ266200; ITS-V48I: DQ266223); *V. dahliae* VCG1A (Act-V138I: DQ266108; Bt-V138I: DQ266129; V138I-cal: DQ266154; H3-V138I: DQ266180; ITS-V138I: DQ266204); *V. dahliae* VCG1B (Act-607I: DQ266111; Bt-V607I: DQ266131; V607I-cal: DQ266156; H3-V607I: DQ266182; ITS-V607I: DQ266206); *V. dahliae* VCG2A (Act-320I: DQ266123; Bt-V320I: DQ266142; V320I-cal: DQ266177; H3-V320I: DQ266194; ITS-V320I: DQ266218); *V. dahliae* VCG2B^{334} (Act-613I: DQ266114; Bt-V613I: DQ266132; V613I-cal: DQ266158; H3-V613I: DQ266183; ITS-V613I: DQ266207) (the sequences for 2Ba were identical to 2B^{334}); *V. dahliae* VCG2B^{824} (Act-357I: DQ266113; Bt-V357I: DQ266133; V357I-cal: DQ266155; H3-V357I: DQ266184; ITS-V357I: DQ266208); *V. dahliae* VCG4A (Act-131-M: DQ266120; Bt-131-M: DQ266145; 131-M-cal: DQ266168; H3-131-M: DQ266197 and ITS-131-M: DQ266221); *V. dahliae* VCG4B (Act-304I: DQ266116; Bt-V304I: DQ266140; V304I-cal: DQ266157; H3-V304I: DQ266192; ITS-V304I: DQ266216); *V. dahliae* VCG6 (Act-560I: DQ266118; Bt-V560I: DQ266139; V560I-cal: DQ266167; H3-V560I: DQ266190; ITS-V560I: DQ266214).

Phylogenetic analyses were performed independently for *Act*, *β-tub*, *Cal*, *H3* and ITS loci. Thus, sequences from each of the five *loci* were aligned using the CLUSTALV method implemented in the MegAling software (DNAStar Inc, Madison, WI). All DNA sequences were analyzed independently by neighbour-joining (Saitou & Nei 1987) using TREECON v.1.3b software (Van der Peer 2003) and genetic distances (Jukes & Cantor 1969), with indels being considered as changes in the sequence. All trees were rooted using gene sequences of *Verticillium nigrescens* V51. A bootstrap analysis of 1000 permutations was used to test reliability of branches in the tree.

A similar phylogenetic analysis of the *V. dahliae*-specific polymorphic sequence was conducted with TREECON v.1.3b software. All sequences used in this analysis but the new *seq6* identified in this work were previously reported to be either associated to different *V. dahliae* VCGs or present in different VDLSP isolates (Collins *et al.* 2003, 2005; Collado-Romero *et al.* 2008). Thus, *seq1* is present in VCGs 2B^{824} and 6 (AN: DQ266246); *seq2* in VCGs 2A and 4B (AN: DQ266247); *seq3* is amplified in VDLSP isolates belonging to AFLP group α, AN:DQ266248; *seq4* in VCGs 1A, 1B and 2B^{334} (AN: DQ266249); *seq5* correspond to VDLSP strain MD73 belonging to AFLP group β (AN: AF363244); and *seq7* is amplified in VCG4A isolate 131-M (AN: DQ266245).

Results

AFLP phylogenetic analysis of Verticillium *spp. isolates*

All *Verticillium dahliae* isolates previously analyzed clustered with their VCG as predicted according to Collado-Romero *et al.* (2006) (Fig 1). Similarly, AFLP phylogenetic analysis grouped VDLSP isolates in a single cluster. Moreover, genetic distances placed VDLSP isolates more related to *V. dahliae* than to *Verticillium albo-atrum* although they were clearly differentiated from isolates of these two species (Fig 1). Interestingly, the *V. dahliae* VCG3 isolate (70-21) appeared as unrelated to *V. dahliae* isolates as to *Verticillium nigrescens*, *V. albo-atrum* or VDLSP isolates.

Characterization of Verticillium *spp. gene sequences*

Primer pairs used for amplification of *Act*, *β-tub*, *Cal* and *H3* genes, and the ITS regions amplified at least one DNA sequence in all *Verticillium* spp. isolates assayed. A BLAST searching in the GenBank database let identify the amplified sequences as corresponding to the expected genes.

From the *Verticillium nigrescens* isolate V51 a single sequence (*ca.* 250 bp) was amplified for the *Act* gene (Act-V51I, AN: DQ266105). On the other hand, two different *Act* sequences were amplified from the *Verticillium dahliae* VCG3 isolate 70-21: one of *ca.* 200 bp, sequence Act-70-21_b, (AN: DQ266107)

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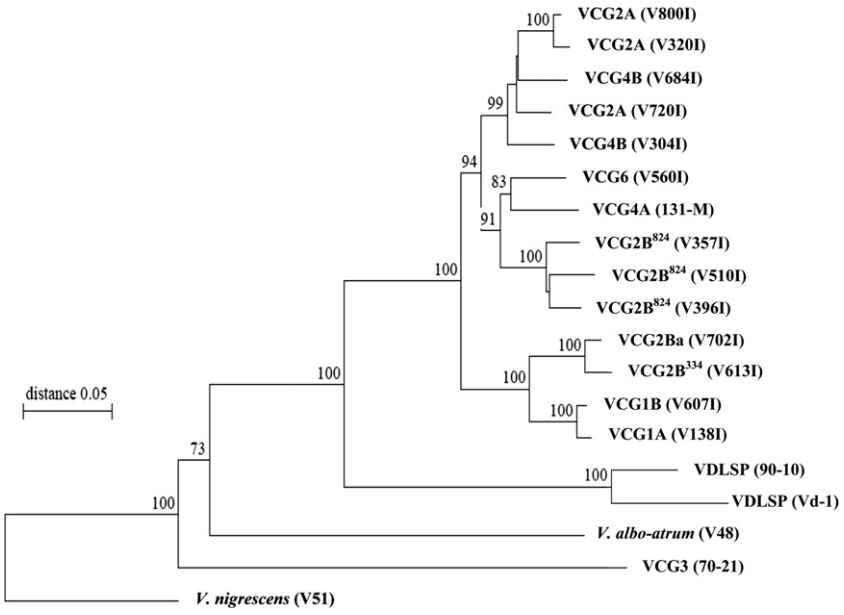

Fig 1 – Phylogenetic tree among *Verticillium* spp. inferred from AFLP markers with TREECON v.1.3b software (Van de Peer & de Wachter 1994). Genetic distances were computed by the simple matching method (Sneath & Sokal 1973) and derived from neighbour-joining (Saitou & Nei 1987) analysis of 652 AFLP markers. Isolate codes are shown in parentheses. VDLSP means *Verticillium dahliae* var. *longisporum*. *V. dahliae* isolates are named according to their VCG (vegetative compatibility group) belonging. Bootstrap values above 70 % are shown (1000 replicates). *Verticillium nigrescens* AFLP profile was used to root the tree.

that showed 100 % identity to that one present in all *V. dahliae* isolates; and a different *Act* sequence of 325 bp (Act-70-21_a, AN: DQ266106). This latter sequence (Act-70-21_a) showed less than 50 % identity with all *Verticillium* spp. *Act* sequences here characterized, and even less identity with others available in GenBank. Thus, maximum identities were found with *Act* sequences from *Phaeoacremonium* spp., and *Epichloe* spp., although they covered less than 31 % of the total sequence. Amplifications of the *Act* gene from VDLSP isolates yielded a single electrophoretic band of *ca.* 200 bp. However, sequencing of this PCR product repeatedly showed ambiguous chromatograms at certain nucleotide positions (two nucleotides in the same position in the chromatograms) suggesting the presence of mixed sequences. Therefore, the amplified bands of isolates Vd-1 and 90-10 were cloned and inserts of four independent transformants per isolate were sequenced. In total, four different *Act* sequences were found (Act-VDLSP_A, AN: DQ266128; Act-VDLSP_B, AN: DQ266125; Act-VDLSP_C, AN: DQ266126; Act-VDLSP_D, AN: DQ266127). Only Act-VDLSP_A was present in the two isolates, the others were only in one of them (Fig 2). None of these sequences showed 100 % identity with either *Verticillium* spp. *Act* sequences or other present in GenBank. Three of these *Act* sequences, Act-VDLSP_A, VDLSP_C and VDLSP_D, were obtained from the single isolate Vd-1.

Amplification of β-*tub* gene fragment yielded a sequence of 546 bp (Bt-V51, AN: DQ266148) from the *V. nigrescens*. As for the *Act* sequences, direct sequencing of the unique PCR amplified from VCG3 isolate product unraveled the presence of several sequences according to the chromatograms obtained. Two different sequences were identified from the *V. dahliae* VCG3 isolate after cloning and sequencing. One of these two sequences (i.e. 70-21seq-b, 448 bp, AN: DQ266153) was identical to a sequence previously identified in all *V. dahliae* VCGs except VCG6 (Collado-Romero *et al.* 2008); the other showed one single nucleotide polymorphism (Bt-70-21seq-a, AN: DQ266146). Similarly, direct sequencing of the unique PCR product (*ca.* 448 bp) obtained from VDLSP isolates showed mix of sequences in the chromatograms for the putative β-*tub* gene. Cloning of the multiple inserts present in the single electrophoretic band (using isolates Vd-1 and 90-10) and subsequent sequencing of four transformants obtained from each VDLSP isolate yielded four polymorphic sequences (Bt-VDLSP_A, 549 bp, AN: DQ266149; Bt-VDLSP_B, 546 bp, AN: DQ266150; Bt-VDLSP_C, 546 bp, AN: DQ266151; and VDLSPBt_D, 546 bp, AN: DQ266152). Similarly to the *Act* gene, none of these putative β-*tub* sequences were identical to *V. dahliae*, *Verticillium albo-atrum* or *V. nigrescens* β-*tub* gene sequences. Moreover, three of these sequences were identified in one of the VDLSP isolates (90-10); and the sequence Bt-VDLSP_A was present in the two isolates.

For *Cal* gene, a fragment of 480 bp was amplified from the *V. nigrescens* V51 (Cal-V51, AN: DQ266164). Similarly, PCR and direct sequencing from *V. dahliae* VCG3 isolate 70-21 yielded a single sequence of 492 bp (Cal-70-21, AN: DQ266173), but that was different from any *Cal* gene sequence previously identified in *V. dahliae* VCGs (Collado-Romero *et al.* 2008) or from other *Verticillium* spp. (identity range with *Verticillium* spp. *Cal* sequences 46.1–48.1 %). A BLAST search using this sequence failed to identify any closely related sequences. The best alignments were found with *Cal* sequences from *Aspergillus* spp. (AN: EF661175.1) and *Sporothrix* spp. (AN: AM490363.1), but the total coverage of the identities was less than 56 %. As for the previous genes, VDLSP isolates showed single bands (*ca.* 520 bp) in agarose gels, the subsequent DNA cloning and

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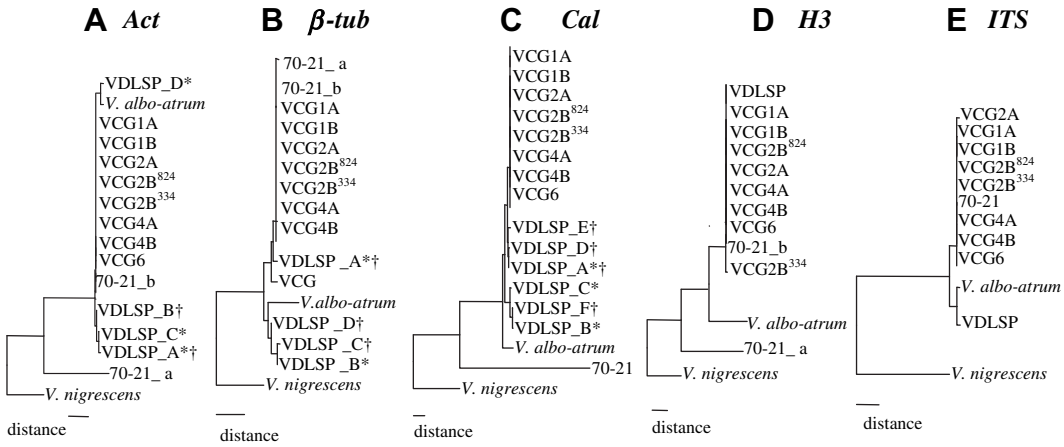

Fig 2 – Gene trees for actin (*Act*, A), β-tubulin (β-*tub*, B), calmodulin (*Cal*, C), histone 3 (*H3*, D) and ITS region of rDNA (ITS, E) loci. Trees were inferred with TREECON v.1.3b software (Van de Peer & de Wachter 1994) using genetic distances calculated according to Jukes & Cantor (1969) and the neighbour-joining method (Saitou & Nei 1987). Indels were considered as changes in the sequence. Sequences of *Verticillium nigrescens* isolate were used to root all trees. The scale bar below each phylogram represents a single character change. VDLSP, *Verticillium dahliae* var. *longisporum* sequences. VDLSP_A–VDLSP_F, *V. dahliae* var. *longisporum* polymorphic alleles for each gene; * and †, alleles that have been simultaneously found in *V. dahliae* var. *longisporum* Vd-1 or 90-10, respectively. 70-21_a and 70-21_b, polymorphic sequences found simultaneously for each gene in *V. dahliae* VCG3 isolate 70-21.

sequencing of which revealed that each band comprised multiple PCR products. Indeed, six polymorphic sequences were identified for the putative VDLSP *Cal* gene (Cal-VDLSP_A, AN: DQ266169; Cal-VDLSP_B, AN: DQ266170; Cal-VDLSP_C, AN: DQ266171; Cal-VDLSP_D, AN: DQ266172; Cal-VDLSP_E, AN: DQ266179; Cal-VDLSP_F, AN: DQ266178). Four of them were in isolate 90-10 (Cal-VDLSP_A, D, E and F, Fig 2), and sequence Cal-VDLSP_A was present in both isolates. As for the previous conserved genes, none of the VDLSP *Cal* sequences were identical to *V. dahliae*, *V. albo-atrum*, or *V. nigrescens* *Cal* gene sequences.

The *V. nigrescens* isolate amplified a sequence of 442 bp for the *H3* gene fragment (H3-V51, AN: DQ266201). Again, two different sequences were identified in the *V. dahliae* VCG3 isolate 70-21, one sequence of 439 bp (H3-70-21seq_b, AN: DQ266199) and another one of 388 bp (H3-70-21seq_a, AN: DQ266198). Sequence H3-70-21seq_b was identical to *H3* sequences found in all *V. dahliae* VCGs but VCG2B^{334} (Collado-Romero *et al.* 2008), whereas sequence H3-70-21seq_a had maximum identities with *Calonectria* spp. (88 % identity, 97 % sequences overlapped) and *Cylindrocladium* spp. (87 % identity, 95 % sequences overlapped) *Cal* gene sequences. Contrary to that found for the other genes, VDLSP isolates only amplified one product for their putative *H3* genes (ANs: DQ266202 for H3-V558, and DQ266203 for H3-V559), which sequence was identical to that one found in all *V. dahliae* isolates but those in VCG2B^{334}.

Finally, amplification of the ITS-1 and -2 from *V. nigrescens* V51 yielded a sequence of 466 bp (ITS-V51, AN: DQ266224). This sequence was the most divergent with regards to all *V. dahliae*, *V. albo-atrum*, *Verticillium tricorpus* or *Verticillium nubilum* ITS sequences either reported in this work or previously deposited in the databases. The unique sequence amplified from *V. dahliae* VCG3 isolate 70-21 (452 bp; ITS-70-21, AN: DQ266222) was identical to that found in *V. dahliae* VCGs except VCG2A (Collado-Romero *et al.* 2008). Only one ITS sequence was amplified from VDLSP isolates included in this study (ANs: DQ266225 [isolate Vd-1], DQ266226 [isolate 90-10]). This VDLSP ITS sequence was different from both *V. dahliae* and *V. albo-atrum*. However, these sequences showed more identity (99.5 %) with *V. albo-atrum* ITS sequences (one single nucleotide polymorphism, SNP) than with *V. dahliae* ITS sequences (98.3 %).

Phylogenetic analysis of conserved DNA sequences

Phylogenetic trees for each conserved gene and the ITS region studied in this work are shown in Fig 2. In all cases, *Verticillium nigrescens* sequences were the most divergent within the *Verticillium* genus. *Verticillium albo-atrum* differed from *Verticillium dahliae* and VDLSP isolates, although it was more closely related to these taxa than to *V. nigrescens*. The phylogenetic relationship of the *V. dahliae* VCG3 isolate (70-21) with respect to the other *Verticillium* spp. was interesting and unexpected. One of the two sequences identified in this isolate for *Act* and *H3* genes (i.e. sequence 70-21_b), as well as the two *Bt* sequences and the ITS sequence, grouped isolate 70-21 with the corresponding *V. dahliae* homologue sequences (Fig 2A, B, D and E). In contrast, the second *Act* and *H3* sequences (70-21_a) (Fig 2A and D), and the only *Cal* sequence (Fig 2C) identified in isolate 70-21 were as unrelated to *V. dahliae* homologues as to *V. albo-atrum* or VDLSP sequences.

With regard to conserved genes in VDLSP isolates, some of the sequences (alleles) grouped these isolates next to (e.g. Act-VDLSP_B, Bt-VDLSP_A) or among *V. dahliae* sequences (H3-VDLSP, Fig 2D); however, other sequences grouped them next to *V. albo-atrum* (e.g. Act-VDLSP_D, Bt-VDLSP_D or ITS-VDLSP) (Fig 2A, B, D

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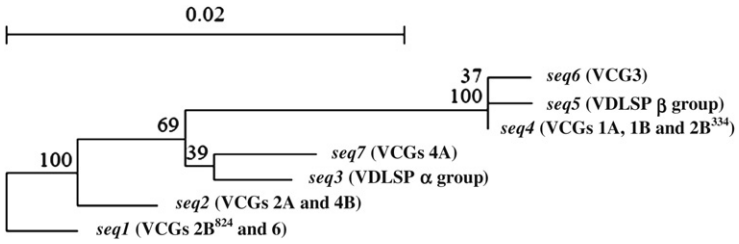

Fig 3 – Phylogenetic tree obtained for the polymorphic *Verticillium dahliae*-specific sequences (*sequences* 1–7) using TREECON v.1.3b software (Van de Peer & de Wachter 1994). *Sequences* 1, 2, 3, 4, 5 and 7 were reported in previous works (Collado-Romero *et al.* 2008; Collins *et al.* 2005). *Sequence* 6 has been characterized for the first time in this work. In brackets, sequence type-VCGs/VDLSP group correspondence is shown. VDLSP α and VDLSP β correspond to the division among *V. dahliae* var. *longisporum* isolates proposed by Collins *et al.* (2005). Isolates used in this work belonged to VDLSP α group.

and E). Nevertheless, none of the VDLSP sequences were identical to the *V. albo-atrum* homologue. Additionally, two groups of sequences could be identified in those conserved genes for which we found multiplicity of sequences/alleles (i.e. *Act*, *Bt* and *Cal* genes) (Fig 2A–C).

Analysis of the polymorphic Verticillium dahliae*-specific DNA sequence*

The two VDLSP isolates used in this study amplified a PCR product with identical nucleotide sequence as that previously identified in the group α of VDLSP isolates and denoted as *seq3* (Fig 3) (Collins *et al.* 2005). On the other hand, *Verticillium dahliae* VCG3 isolate 70-21 amplified a newly reported sequence of 543 bp (AN: DQ266245), which is hereby denoted as *seq6*. This sequence is different to those previously reported in other *V. dahliae* VCGs or VDLSP isolates (Collins *et al.* 2005; Collado-Romero *et al.* 2008). Moreover, the new *seq6* showed more related to *seq4* (found in *V. dahliae* isolates of VCG1 and VCG2B^{334}) and *seq5* (found in VDLSP isolates of the group β) (Collins *et al.* 2003, 2005) than to sequences found in other VCGs (*seq1*, *seq2* or *seq7*) or in other VDLSP isolates (*seq3*) (Fig 3).

Discussion

In previous studies we used AFLP fingerprinting to study the genetic diversity existing within populations of *Verticillium dahliae* (Collado-Romero *et al.* 2006), and further demonstrated that the use of phylogenetic analysis of selected DNA sequences and AFLP markers was useful to establish phylogenetic relationships at the intraspecific level (i.e. VCGs) within those populations (Collado-Romero *et al.* 2008). Here, we have applied the analysis of selected DNA sequences in order to unravel the phylogenetic relationship of two representative isolates of the controversial species *Verticillium longisporum* (isolates 90-10 and Vd-1) (Zeise & von Tiedemann 2001; Collins *et al.* 2003, 2005; Fahleson *et al.* 2004; Pantou *et al.* 2005; Clewes *et al.* 2008; Collado-Romero *et al.* 2008) with other phytopathogenic members of the *Verticillium* genus (*V. dahliae*, *Verticillium albo-atrum*) and with *Verticillium nigrescens*. *V. nigrescens* has been recently described as not congeneric with *Verticillium* spp. and reassigned to *Gibellulopsis* genus (*Gibellulopsis nigrescens* (Pethybr.) Zare, W. Gams & Summerb) (Zare *et al.* 2007). Our phylogenetic results would support a lower relativeness among this isolate (V51) and the other *Verticillium* spp. Likewise, the same approach was conducted to study the phylogenetic position of the VCG3 within *V. dahliae*, using the isolate which is used as the international reference tester for assignment of *V. dahliae* isolates to this VCG (Joaquim & Rowe 1991; Korolev *et al.* 2001; Collado-Romero *et al.* 2006, 2008; Jiménez-Díaz *et al.* 2006).

Results from the present study and of previous phenetic analysis of AFLP patterns from the VDLSP isolate 90-10 (Collado-Romero *et al.* 2006) indicate that isolates Vd-1 and 90-10 are molecularly divergent from *V. dahliae*, *V. albo-atrum* or *V. nigrescens* (Fig 1). These data might support these VDLSP isolates constituting as a different species from *V. dahliae* or *V. albo-atum*, in agreement with Karapapa *et al.* (1997) and Pantou *et al.* (2005). However, the AFLP fingerprinting, mainly due to the homoplasy of this type of marker, was not enough to unravel the hybrid origin of VDLSP isolates as well as to identify their putative parents. Nevertheless, our findings here reported are in full agreement with Clewes *et al.* (2008), who reported the presence of multiple (two or three) sequences for the β-*tub* gene in a set of VDLSP isolates (denoted by these authors as long-spored *V. dahliae* isolates) while this present paper was in preparation. Interestingly, the β-*tub* gene sequence was also used by Qin *et al.* (2006) to explore phylogenetic relationships among *Verticillium* species, including *V. dahliae* and VDLSP isolates. However, these authors did not report β-*tub* allele multiplicity in any of the VDLSP isolates used, presumably because they purified PCR products and cloned them prior to sequencing or, alternatively, a low number of clones were sequenced. In view of our results, we believe that this later caveat is of much importance and should be considered in further studies of similar nature. Thus, we consider that an adequate strategy for this type of study should be, firstly, sequencing of the PCR product previous to cloning, and subsequently, cloning of the amplicons and sequencing of a fair, representative number of inserts.

In this present study, the interspecific hybrid origin of at least the two VDLSP isolates (90-10 and Vd-1) was even more strongly supported by the multiplicity of sequences found in two other conserved genes (*Act* and *Cal*) besides that reported for the β-*tub* gene. The presence in both VDLSP isolates analyzed of an *H3* sequence identical to *V. dahliae* supported this species as one of the parental. However, although none of the

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sequences in this work analyzed were identical to *V. albo-atrum* ones, ITS sequence was more related to *V. albo-atrum* than to *V. dahliae*. Additionally, the analysis of the *V. dahliae*-specific sequence (Fig 3) let to infer some conclusions about putative parents of these hybrid VDLSP isolates. Although a previous study indicated a close relationship among *seq*5 present in VDLSP β group isolates and *V. dahliae* isolates in VCG1 and $2B^{334}$ (Collins *et al.* 2005), in this present study we further identify a closer relationship among VDLSP isolates carrying *seq*3 (α group) and *V. dahliae* isolates of VCG4A. This supports *V. dahliae* as a parental of the α and β groups of VDLSP isolates.

It is worth mention that multiplicity of sequences here found for VDLSP isolates (up to four identified for *Cal* gene in isolate 90-10) could be even greater since we sequenced four colonies per cloning procedure (see Materials and Methods section). Multiplicity of sequences (alleles) with high similarity to those of different species is not only a strong evidence of hybrid origin, but also might indicate a process of genome reorganization likely taking place after the hybridization event. For instance, rearrangement of the hybrid genome after the hybridization event could involve duplication of some DNA regions (for example, *β-tub* or *Act* genes), which subsequently may undergo different evolving processes. These results would support the hypothesis that VDLSP isolates 90-10 and Vd-1 probably arose from a rather ancient hybridization event. Moreover, for genes for which several VDLSP sequences were found, i.e. *Act*, β-*tub* and *Cal*, they could be differentiated into two groups (Fig 2A–C), which would be in agreement with two distinct, parental sequences.

The study on phylogenetic relationships among *V. dahliae* VCGs recently reported did not include isolates representative of VCG3 (Collado-Romero *et al.* 2008). Unexpectedly, the AFLP analysis of *V. dahliae* VCG3 isolate 70-21 carried out in the present study did not group this isolate within or close to any of VCG/AFLP group previously described (Collado-Romero *et al.* 2006, 2008). Moreover, that isolate showed as much dissimilarity with isolates representative of *V. dahliae*, VDLSP or *V. albo-atrum*. In contrast to VDLSP isolates, isolate 70-21 is able to form *nit* mutants and therefore has been widely used as international reference tester in a number of genetic studies of *V. dahliae* (Collado-Romero *et al.* 2006, 2008; Joaquim & Rowe 1991; Jiménez-Díaz *et al.* 2006). This isolate is assumed to be haploid but, to the best of our knowledge no studies have provided any firm evidence of that. The presence of two alleles for each of genes *Act*, β-*tub* and *H3* in the VCG3 isolate clearly suggested a hybrid origin for isolate 70-21. Gene genealogies of one of those alleles suggest a *V. dahliae* isolate as putative parent. Since VCG3 isolate 70-21 was originally isolated from potato in the USA (Joaquim & Rowe 1991), that parent would likely be a VCG1 isolate (Figs 2 and 3), which is a more extensively distributed VCG compared to the alternative parent, $VCG2B^{334}$, which has only been identified in a localized region in Spain (Jiménez-Díaz *et al.* 2006; Collado-Romero *et al.* 2006, 2008). Moreover, the PCR molecular patterns pointed to VCG1B isolates as a putative parent of VCG3 isolates because they share the PCR ''pattern B'' (Table 1), which is not present in VCG1A isolates from USA (Collado-Romero *et al.* 2006, 2009). In contrast, nothing could be concluded about the other parental of this apparent hybrid according to the results here presented and the sequences currently available in the databases. Previous studies suggested that some haploid *V. dahliae* isolates could have emerged from diploid isolates (i.e. VDLSP) undergoing haploidization events (Collins *et al.* 2003; Clewes *et al.* 2008). In any case, the hereby proposed hybrid origin of *V. dahliae* VCG3 70-21 does not appear related to the VDLSP isolates of this study as showed by gene phylogenies (Fig 2). On the other hand, it is worth mention that, in addition to the molecular differences here identified among VDLSP and the VCG3 isolates, there is an important difference between these two groups with regard to the frequency of isolation from infected crops. Indeed, VDLSP isolates seem to have naturally exploited and fitted adequately to its biological niche (mainly crucifer crops) probably benefiting from some selective advantage (Karapapa *et al.* 1997; Zeise & von Tiedemann 2001, 2002a, b; Barbara & Clewes 2003; Fahleson *et al.* 2004). Conversely, few reports are available about host preference of VCG3 isolates, which in fact are rarely isolated from infected crops (Joaquim & Rowe 1991; Elena & Paplomatas 1998). Nevertheless, an adequate characterization of this isolate is of great importance since, as previously stated, it has been broadly used as reference tester for assignment of *V. dahliae* isolates to VCG3.

The results from this study support the occurrence of hybridization events giving rise to enhanced diversity within the *Verticillium* genus. In the case of VDLSP isolates examined in this study, a *V. dahliae* isolate and a closely related taxon to *V. albo-atrum* are pointed out as their ancestors. The lack of homogeneity within VDLSP isolates reported in previous studies (Collins *et al.* 2003, 2005; Zeise & von Tiedemann 2002b; Clewes *et al.* 2008) might not allow to include all these isolates within a unique different taxon (*V. longisporum*). Nevertheless, considering VDLSP isolates as *V. dahliae* as suggested by Klosterman *et al.* (2009) would not be adequate for the isolates here analyzed either. Thus, a broader scrutiny of the genetic diversity within the VDLSP group, making use of the approach used in our study, would be very useful to finally clarify the proper taxonomic status of isolates within that group. With regards to *V. dahliae* VCG3 isolate 70-21, putative parents could be a *V. dahliae* isolate and an individual of a different (likely non-*Verticillium*) species. Results from gene phylogenies also suggested the possibility that isolate 131-M of *V. dahliae* VCG4A (obtained from potato in North America) might have arisen from a hybridization event between members of two different *V. dahliae* VCGs (Collado-Romero *et al.* 2008). Similarly, Clewes *et al.* (2008) identified a haploid *V. dahliae* isolate (MD71 isolated from chamomile in Germany) that could have emerged after haploidization of a VDLSP isolate. Altogether, these results suggest that parasexuality can be an important mechanism, and maybe not infrequent, contributing to diversity and evolution within the genus *Verticillium*. This possibility is of significance for the potential emergence of new pathogenicity traits such as new host adaptation and/or enhanced virulence to present host genotypes.

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